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HAZARD EVALUATION DIVISION

STANDARD EVALUATION PROCEDURE

ACUTE TOXICITY TEST FOR ESTUARINE AND MARINE ORGANISMS

(Mollusc 96-Hour Flow-Through Shell Deposition Study)

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16. Abstract (Limit: 200 words)

The Standard Evaluation Procedure (SEP) for the Mollusc 96-Hour Flow-Through Shell Deposition Test is a guidance document primarily intended for Agency reviewers and the regulated industry who evaluate ecological effects data specified in 40 CFR Part 158.145. The SEP is also intended to provide information to the general public indicating how the Agency evaluates these types of studies. As such, it is designed to supplement Subdivision E of the Pesticide Assessment Guidelines: Hazard Evaluation - Wildlife and Aquatic Organisms. This SEP provides an Introduction, Materials and Methods, Reporting Requirements, Reviewer Evaluation, and Appendix of appropriate methods to guide the review and scientific evaluation of pesticide effects on molluscs.

The SEP for the Mollusc 96-Hour Flow-Through Shell Deposition Test is only one of a number of SEP's published by the National Technical Information Service as a supplement to Subdivision E of the Pesticide Assessment Guidelines.

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STANDARD EVALUATION PROCEDURE

PREAMBLE

This Standard Evaluation Procedure (SEP) is one of a set of guidance documents which explain the procedures used to evaluate environmental and human health effects data submitted to the Office of Pesticide Programs. The SEPs are designed to ensure comprehensive and consistent treatment of major scientific topics in these reviews and to provide interpretive policy guidance where appropriate. The Standard Evaluation Procedures will be used in conjunction with the appropriate Pesticide Assessment Guidelines and other Agency Guidelines. While the documents were developed to explain specifically the principles of scientific evaluation within the Office of Pesticide Programs, they may also be used by other offices in the Agency in the evaluation of studies and scientific data. The Standard Evaluation Procedures will also serve as valuable internal reference documents and will inform the public and regulated community of important considerations in the evaluation of test data for determining chemical hazards. I believe the SEPs will improve both the quality of science within EPA and, in conjunction with the Pesticide Assessment Guidelines, will lead to more effective use of both public and private resources.

John W. Melone

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ACUTE TOXICITY TEST FOR ESTUARINE AND MARINE ORGANISMS: MOLLUSC 96-HOUR FLOW-THROUGH SHELL DEPOSITION STUDY

I. INTRODUCTION

A. When Required

Acute toxicity studies with mollusc are required to support registration of an end-use product intended for direct application to the estuarine or marine environment. These studies are also necessary when it is expected that the pesticide would enter this environment in significant concentrations because of its expected use or mobility.

B. Purpose

- To establish acute toxicity levels of the active ingredient to nontarget marine and estuarine organisms;
- To compare toxicity information with measured or estimated pesticide residues in the estuarine or marine environment to assess potential impact to invertebrates;
- To provide support for precautionary label statements to minimize adverse effects to estuarine or marine nontarget organisms; and
- To indicate the need for further testing and/or field studies.

C. Test Material

1. Technical Grade

Tests must be conducted with the technical grade of the active ingredient. If more than one active ingredient constitutes a technical product then the technical grade of each active ingredient must be tested separately.

2. End-Use Product

The applicant may be required to test the end-use product as well if:

- The end-use product will be introduced directly into the marine or estuarine environment when used as directed;
- The mollusc EC_{50} of the technical grade of the active ingredient is equal to or less than the expected environmental concentration in the marine or estuarine environment when the end-use product is used as directed;

- An ingredient of the end-use product is expected to enhance the toxicity of the end-use product beyond that expected from the active ingredient(s); or,

- The technical product is insoluble in water but the formulated product is soluble in water. In that case the carriers or inerts of the formulated product must be tested in a solvent control.

II. MATERIALS AND METHODS: TESTING STANDARDS/RECOMMENDATIONS

A. Acceptable Protocols

The EEB does not endorse any one protocol. It is sometimes necessary to alter the procedures presented in published protocols to meet the needs of the chemicals or organisms used. However EEB does recommend some protocols as guidance for developing mollusc shell-deposition toxicity test protocol. These references include:

American Public Health Association, American Water Works Association and Water Pollution Control Federation. 1981. Standard Methods for the Examination of Water and Wastewater. Fifteenth edition. Publication office: American Public Health Association, 1015 18th Street NW, Washington, D.C., 20036.

Anonymous. 1976. Bioassay Procedures for the Ocean Disposal Permit Program. Environmental Research Laboratory, Gulf Breeze, USEPA. EPA-600/9-78-010.

American Society for Testing Materials. 1980. Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians. E 729-80. Published by ASTM Committee on Standards, 1916 Race Street, Philadelphia, PA, 19103.

These referenced protocols are presented as flexible guidance to help researchers design scientific protocol and to help the reviewer validate studies. It is important to recognize that tests are validated based on whether they provide scientifically sound information on the acute toxicity of the test material to molluscs that is useful in risk assessment and whether they fulfill guideline requirements. This is more important than whether a referenced protocol was followed step-by-step.

B. Test Organisms

1. Acceptable Species

The selected species should have a demonstrated sensitivity to known toxicants. If possible they should be species that occur in the area of exposure or that represent exposed species.

Numerous species are specifically referenced in "Methods of Acute Toxicity Tests with Fish, Macroinvertebrates, and Amphibians" (Committee on Methods for Toxicity Tests with Aquatic Organisms, 1975 and ASTM E 729-80). The following species are preferred because there is a substantial data base available on them:

Crassostrea gigas	Pacific oyster
Crassostrea virginica	Eastern oyster

Some other species may be acceptable if they have been shown to be sensitive in acute toxicity tests.

2. Size/Age/Physical Condition

The molluscs used for this test should be young. Oysters, for example, should be about 25 to 50 mm in height (i.e., the long axis) with a reasonably flat, rounded shape. Small individuals are preferred because they are less affected by the temperature. That is, they continue to grow in spite of temperature fluctuations as opposed to older individuals that tend to become less active at temperature extremes.

Test molluscs must be observed prior to testing for signs of disease, stress, physical damage, and mortality. Injured, dead, and abnormal individuals must be discarded. If the organisms show signs of disease or stress, remedial action must be taken during the acclimation period and not during the test period. Treated organisms may be used for testing as long as they no longer exhibit signs of disease.

3. Source/Acclimation

All organisms must be from the same source. This may include laboratory or commercial stocks. Organisms captured in the wild are acceptable provided they meet the requirements pertaining to physical condition and age/size criteria mentioned above.

After collection, molluscs must be held for at least ten days for observation and acclimation. To avoid unnecessary stress, rapid changes in water temperature and quality must be avoided. Feeding, handling, and other maintenance must be such that the molluscs are not stressed, diseased or damaged. All organisms must be maintained under actual test conditions (temperature and

water quality) for at least 48 hours before the test actually begins.

C. Test Solutions

1. Source of Dilution Water

The dilution water will likely be natural unfiltered seawater as this is a flow-through test. The water should be from an uncontaminated area and of constant quality. That is, the weekly range of salinity is less than 6‰, and the monthly pH range is less than 0.8 of a unit. The water should be unfiltered as the molluscs obtain their food directly from the natural seawater.

2. Temperature

The selected temperature may be between 15° and 30°C, as long as temperature is consistent in all test containers and controls.

D. Testing System

1. Test Vessels

Test containers should be glass or stainless steel. Other materials such as fiberglass or polyethylene may be used since the test is flow-through and the test concentrations are measured.

2. Photoperiod

A 16-hour light and an 8-hour dark photoperiod should be provided, with a 15- to 30-minute transition period between light and dark.

3. Loading

The test containers should be large enough that each individual can sit on the bottom with water flowing freely around it.

4. Solvents

If solvents other than water are necessary, they should be used sparingly, not to exceed 0.5 ml/L in any test solution. The following solvents are preferred:

dimethyl formamide
triethylene glycol
methanol
acetone
ethanol

5. Flow-Through Study

The mollusc 96-hour shell deposition study is a flow-through study.

E. Test Design

1. Test Levels (Nominal/Measured)

Initially, range finding tests may be necessary to define levels of the toxicant needed for definitive studies. Test reports should provide information describing range finding study procedures and results. The information should include sample sizes, test levels, and mortality data.

If it can be shown (by testing at least 30 individuals) that a chemical will have an EC_{50} greater than 100 ppm, a definitive study need not be performed.

Definitive acute toxicity tests normally are designed to include one or more control groups and a geometric series of at least five toxicant levels to be tested. Each designated treatment group should be exposed to a concentration of toxicant that is at least 60% of the next highest concentration. If a formulated product is tested, it should be clearly stated in the test report whether results are expressed in terms of active ingredient alone or as total formulated product.

2. Number of Test Animals

There should be 20 individuals per test level and in each control.

3. Controls

Each test requires a concurrent control using the same dilution water and the same number of organisms at each test level. If any solvent other than water is used a solvent control should contain the highest concentration of the solvent that was added to any of the test chambers. The control organisms provide the norm for shell deposition that the test organisms will be compared to.

4. Beginning the Test

The test is begun by placing the organisms on the bottom of the test containers of the flow-through system. The toxicant is then injected into the system with a metering system.

5. Measurement of Diluent Characteristics

Temperature should be measured hourly throughout the acclimation and test periods in at least one test chamber.

The pH should be measured at the beginning and end of the test in the control and the high, medium, and low toxicant concentrations.

DO should be measured at the beginning and every 48 hours to the end of the test in the high, medium, and low concentrations. The DO concentrations in each chamber must be between 60% and 100% saturation at all times during the study.

6. Chemical Analysis

It is necessary that the toxicant concentrations in the test solutions be measured at the beginning and at the end of the test to verify the metering system.

III. REPORTING REQUIREMENTS

The test report submitted to the Agency must fully describe the materials and methods used to perform the study. The reviewer must be able to determine from the report that the study was performed under conditions that render the results acceptable for use in a risk assessment and/or for fulfilling a guideline requirement. The following information is particularly important for a complete evaluation.

A. Test Material

If the study is to be performed with the technical grade product, the test material should be clearly identified as to source, batch, and exact purity. Simply identifying the material as technical may not be sufficient because the percent active ingredient of some newer products may increase with time as the manufacturing process is improved to produce greater purity.

For studies involving the end-use product, the exact percent of the active ingredient and the type of formulation (e.g., granular, wettable powder) of the test material should be described. It should clearly state in the test report whether the results are expressed in terms of active ingredient or as formulation.

B. Dilution Water

Test reports submitted to the Agency should include a complete description of dilution waters used in the test. Descriptions should include identification of the source, the chemical characteristics of the water, and information on any pretreatment.

C. Holding of Test Organisms

Test reports should include complete information on holding and acclimation conditions including feeding schedules and treatment for diseases.

D. Observable Effects Criteria

If the peripheral valve edges of a mollusc are mechanically ground away, the mollusc responds by depositing new shell to replace the loss. This 96-hour test is designed to demonstrate the comparative toxicity of test materials based on this response. The growth of new shell is primarily linear during the first week and the rate of deposition is an index of the animals' reaction to ambient water quality. With acceptable water conditions, 25 mm and larger oysters, for example, deposit as much as 1.0 mm of peripheral new shell per day. At the end of the 96-hours, the molluscs are removed and the shell increments measured. The ratio of mean growth of a group of test molluscs to mean growth of control molluscs provides a percentage index of the response of the molluscs to a toxicant concentration.

Test data are independent of minor fluctuations in temperature and salinity during the 96-hour exposure, since the simultaneous shell deposition in control molluscs is considered to be the norm or 100%.

E. Calculated EC50

The statistically calculated EC_{50} (concentration inhibiting shell deposition by 50%) with 95% confidence limits and the method of calculation must be presented. In lieu of a calculated EC_{50}, the study may show that the EC_{50} is greater than 100 ppm. The slope of the dose-response line should be calculated and reported. Some reports have a graphic (log probit/dose level) presentation which is helpful.

F. Temperature/DO/pH

Dissolved oxygen, pH measurements, and the range and average temperature during the study should be reported.

G. Chemical Analysis

If test concentrations were measured, the test report should provide results of analyses. Residues found at the beginning and end of the study should be reported.

H. Testing Protocols

The test report should include reference to the testing protocol(s) used during the study.

IV. REVIEWER'S EVALUATION

A. Review of Test Conditions

The reviewer should identify each aspect of the reported procedure that is inconsistent with recommended protocol. The significance of these deviations must be determined. The number of deviations and their severity will determine the validity of the study and the interpretation of the results.

B. Verification of Statistical Analysis

The reviewer should ensure that the EC_{50} has been properly derived by recalculating these values. An acceptable acute toxicity test should provide not only an EC_{50} but also a NOEL (no observed effect level) and a slope of the dose/mortality response. These data can give further insight into the toxicological characteristics of the chemical such as whether the response is gradual, over a wide range, or rapid.

If the recalculated results differ substantially from the submitted results, the reviewer should note this and attempt to explain the differences.

C. Conclusions

1. Categorization of Results

The significance of inconsistencies in the test procedures must be determined by the reviewer so that the results of the test can be categorized as to whether they fulfill Part 158 regulations and are useful in performing a risk assessment. Categories are described as:

- Core: All essential information was reported and the study was performed according to recommended protocols. Minor inconsistencies with standard methodologies may be apparent; however, the deviations do not detract from the study's soundness or intent. Studies within this category fulfill the basic requirements of current guidelines and are acceptable for use in a risk assessment.

- Supplemental: Studies in this category are scientifically sound, however they were performed under conditions that deviated substantially from recommended protocols. Results do not meet guideline requirements, however the information may be useful in a risk assessment. Some of the conditions that may place a study in a supplemental category include:

- Unacceptable test species;
- Inappropriate test material;
- Concentrations tested were less than 100 ppm but not high enough to produce an effect on the organisms or a precise EC_{50}; and
- Deviations from recommended test solution characteristics (variations in DO, temperature, hardness, and pH can affect toxicological response).

° Invalid: These studies provide no useful information. They may be scientifically unsound, or they were performed under conditions that deviated so significantly from recommended protocols that the results will not be useful in a risk assessment. Examples of studies placed in this category commonly include those where the test system was aerated, test vessels were constructed from materials other than glass, or there were problems of solubility or volatility of the test material. Unless acceptable chemical analyses of actual toxicant concentrations were performed in studies such as these, the reviewer cannot be sure that test organisms were actually exposed to nominally designated concentrations. A study where the test material was not properly identified can also be invalidated.

2. Rationale

Identify what makes the study supplemental or invalid. While all deviations from recommended protocol should be noted, the reviewer is expected to exercise judgment in the area of study categorization.

3. Repairability

Indicate whether the study may be upgraded or given a higher validation category if certain conditions are met. Usually this would involve the registrant submitting more data on the study.

D. Descriptive Classification

The reviewer should indicate what the results were and how much information can be drawn from them. At a minimum, an acute toxicity test will provide an EC_{50} with 95% confidence limits. This should allow classifying the test material based on the following scheme:

EC_{50} (ppm)	Category Description
< 0.1	very highly toxic
0.1 - 1	highly toxic
1 - 10	moderately toxic
10 - 100	slightly toxic
> 100	practically non-toxic

These descriptive categories are for inter-chemical comparison only and do not reflect actual environmental risk to the test organism. The results may provide other useful information such as slope or a no observed effect level (NOEL). The NOEL would be the highest concentration at which no observable effects occurred. These additional data are useful in a risk assessment.

E. References

The reviewer should reference any information used in the validation procedure. This should include protocol documents, statistical methods, or information taken from files of other branches.